RP-HPLC Stability Indicating Assay Technique Development and Validation for the Simultaneous Evaluation of Fenpiverinium, Pitofenone and Diclofenac

Syed Ibrahim Baje[1], B.Jyothi2, N.Madhavi3[*]

[1,3] PG department of chemistry, JKC college,Guntur,A.P.

[2] Department of chemistry Swarna Bharathi institute of Science and Technology, Khammam, Telangana.

Abstract: A tranquil, meticulous new approach has been developed for the simultaneous evaluation of the Fenpiverinium, Pitofenone and Diclofenac in bulk and tablet dosage form. Chromatography was carried out on an Discovery 250mm x 4.6 mm, 5µ with a isocratic mobile phase composed of Ortho phosphoric acid and Acetonitrile (50:50A)at a flow rate of 1 mL/min. The column temperature was maintained at 30°C and the detection was carried out using a PDA detector at 241 nm. Validation parameters such as system suitability, linearity, precision, accuracy, specificity, limit of detection (LOD), limit of quantification (LOQ), Stability of sample and standard stock solutions and robustness were studied. The retention time of Fenpiverinium, Pitofenone and Diclofenac were found to be 2.622 min, 3.172 min. and 3.838 min. The percentage recoveries of Fenpiverinium, Pitofenone and Diclofenac were 99.47% 100.30% and 100.02%.The relative standard deviation for assay of tablet was found to be less than 2%. The method is reliable, fast, specific, exact and diplomatic. Hence it is utilized for routine quality control of tablet containing both drugs in quality control laboratories and pharmaceutical industries.

Keywords: ***Ortho phosphoric acid, Acetonitrile*****, system suitability, linearity, precision, accuracy, specificity.**

I. INTRODUCTION

Pitofenone (PIT) (Fig.1) is utilized for Muscles torment antispasmodic found in the advertised definition NOVASPAS, a Bulgarian drug [1] in blend with Fenpiverinium bromide, and Diclofenac potassium used to ease torment and fits of smooth muscles. The IUPAC name of PIT is Methyl 2-[4-(2-piperidin-1-ylethoxy)benzoyl]benzoate, Molecular equation $C_{22}H_{25}NO_4$, Molecular weight 367.45 g/mol. It has been approved by HPLC, Ion match fluid chromatography and by UV Spectrophotometry.

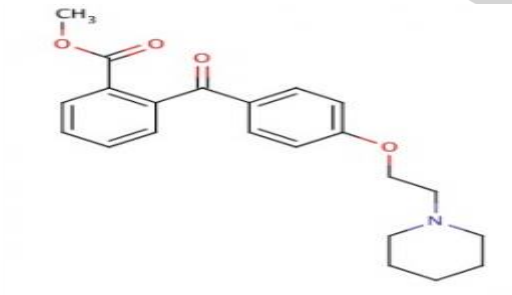

Fig.1. Chemical structure of Pitofenone

Fenpiverinium bromide (FEN) (Fig.2) is an anticholinergic,anti muscarinic and antispasmodic compound; [2] combined with Pitofenone hydrochloride and helps in treating spams of smooth muscles and muscular pain[3,4].The IUPAC name of artificially 1-(4-amino-4-oxo-3,3-diphenylbutyl)- 1-methylpiperidinium, Molecular equation $C_{22}H_{29}N_2O$, Molecular weight 417.391 g/mol. Fenpiverinium bromide is legitimate in United States of Pharmacopoeia.[5] Techniques like UV method[6] and extraction spectrometry[7] are assisted for the measurement of Fenpiverinium bromide.

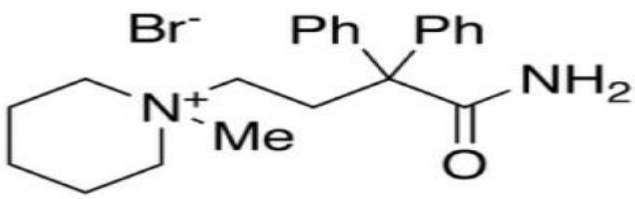

Fig.2. Chemical structure of Fenpiverinium bromide

Diclofenac (DIC) (Fig.3) is accessible as sodium and potassium salt frame in different marketed dosage forms. The fundamental contrast between the two is that diclofenac potassium is assimilated into the body more rapidly than diclofenac sodium. A fast activity is valuable where quick torment help is required. The IUPAC name of diclofenac is [(2,6-dicholoroanilinol)]-phenyl acetic acid. It is a sodium or potassium salt of an arylacetic corrosive subsidiary. It represses prostaglandins amalgamation by meddling with the activity of prostaglandin synthetase (Cyclooxygenase)[8]. It has analgesic, anti-incendiary, anti-inflammatory and antipyretic movement. It is broadly utilized as a part of different ailments including rheumatoid joint inflammation and osteoarthritis, delicate tissue issue, renal colic, intense gout, headache and dysmenorrhea[9]. UV spectrophotometric methods [10-12] and superior fluid chromatography (HPLC) [13-14] are frequently utilized in the investigation of diclofenac. Numerous HPLC

techniques with UV recognition were embraced in the writing for the assurance of DIC [15-21].

In the present work, a security demonstrating, straightforward, fast, exact and delicate turn around stage HPLC strategy was supported and approved for the synchronous estimation of Pitofenone (PIT), Diclofenac potassium(DIC) and Fenpiverinium bromide(FEN) in pharmaceutical tablet measurement frame.

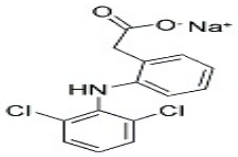

Fig.3.Chemical structure of Diclofenac Potassium

A new approach of RP-HPLC has been developed for validation and evaluation of method for estimation and validation of Fenpiverinium, Pitofenone and Diclofenac in formulation in accordance with the ICH guidelines[6-10]

II. EXPERIMENTAL

*Instrumentation***:** Chromatography was analysed with Alliance waters 2695 HPLC provided with high speed auto sampler, column oven, degasser and 2996 PDA detector compacted with class Empower-2 software.

Reagents and chemicals: Fenpiverinium, Pitofenone and Diclofenac were from Spectrum pharma research solutions, Hyderabad. Acetonitrile, Methanol and all other chemicals were obtained from Merck chemical division, Mumbai.Water obtained from Milli-Q water purification system. Commercial formulation (**Novaspas Dosage**: Pitofenone, Diclofenac and Fenpiverinium - 5MG_50MG_0.1MG tablet) were purchased from the local pharmacy.

Chromatographic condition: The chromatographic separation was carried out under the isocratic conditions. Chromatographic separation was achieved by injecting a volume of 10µl of standard into Discovery 250mm x 4.6 mm, 5µ column. The mobile phase composed of Ortho phosphoric acid Acetonitrile (50:50A) was allowed to flow through the column at a flow rate of 1 ml per minute for a period of 7 min at 30^0C column temperature. Identification of the component was carried out at a wavelength of 241 nm. The retention time of the component was found to be 2.622 min, 3.172 min. and 3.838 min for Fenpiverinium, Pitofenone and Diclofenac.

Preparation of 0.1%OPA buffer solution: 1ml of Ortho phosphoric acid was pippeted out and dissolved in a 500ml of Milli-Q water taken in a 1000ml Volumetric flask and final volume was made up to the mark with Milli-Q water.

Preparation of mobile phase: Buffer and acetonitrile are taken in the ratio 50:50 v/v, was degassed in ultrasonic water bath for 10min and filtered through 0.45µ filtered under vacuum filtration.

Preparation of diluent solution: 50 ml of water with is mixed with 50ml of Acetonitrile, in a 1000ml beaker and sonicated for 15min.

Standard Preparation :(Fenpiverinium, Pitofenone and Diclofenac)Accurately weighed 12.5mg of Pitofenone, 0.25mg of Fenpiverinum and 125mg of Diclofenac are transferred to three 25ml volumetric flasks separately. 10ml of methanol was added to flasks and sonicated for 15mins. Flasks were made up with water and acetonitrile (50:50) and labelled as Standard stock solution 1, 2 and 3.From the above stock solution, 1 ml was pipetted out into a 10ml Volumetric flask and then make up to the final volume with diluent. (Fenpiverinium1µg/mL, Pitofenone 50µg/mL and Diclofenac500µg/mL).

***Preparation of Working Standard Solutions*:** Aliquot of 0.25, 0.50, 0.75, 1, 1.25 & 1.5 mL were pipette out from stock solution into 10 mL volumetric flask separately for Fenpiverinium, Pitofenone and Diclofenac and volume was made up to 10 mL with diluent. This gives the solutions of 0.25, 0.5, 0.75, 1, 1.25and 1.50µg/mL for Fenpiverinium, 12.5,25, 37.5, 50, 62.5and 75µg/mL for Pitofenone and 125,250, 375, 500, 625and 750µg/mL for Diclofenac.

Sample preparation:
5 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 1 tablet was transferred into a 10ml volumetric flask, 5ml of diluent added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered solution 1ml was pipette out into a 10 ml volumetric flask and made upto 10ml with diluent. (Fenpiverinium1µg/mL, Pitofenone50µg/mL and Diclofenac500µg/mL)

Method validation:

System suitability tests: It is evaluated by parameters like tailing factor, the number of theoretical plates, retention time.

Linearity: It gives a value proportional to the concentration of component of analyte. By appropriate aliquots of the standard Fenpiverinium, Pitofenone and Diclofenac solution with the mobile phase, six working solutions ranging between 0.25-1.50 µg/mL for Fenpiverinium, 12.5 -7575µg/mL for Pitofenone and 125 - 750µg/mL for Diclofenac were prepared. Each experiment linearity point was performed in triplicate according to optimized chromatographic conditions. The peak areas of the chromatograms were plotted against the concentration of Fenpiverinium, Pitofenone and Diclofenac to obtain the calibration curve.

Accuracy: It gives the true value. To previously analyzed samples of Fenpiverinium, Pitofenone and Diclofenac standard amounts of Fenpiverinium, Pitofenone and Diclofenac corresponding to 50%, 100% and 150% of target concentration were added. The accuracy was expressed as the percentage of analyte recovered by the standard addition method.

Precision: It helps in determining the impurities, determined as repeatability and intermediate precision (ruggedness), and reproducibility. The intra-day and inter-day precision were determined by analyzing the samples of Fenpiverinium, Pitofenone and Diclofenac. Determinations were performed on the same day as well as well as on consequent days.

Limit of detection and the limit of quantification: LOD determines the lowest sample qualitatively and LOQ determines the lowest sample

quantitatively. Limit of detection (LOD) and limit of quantification (LOQ) of Fenpiverinium, Pitofenone and Diclofenac were determined by calibration curve Method. Solutions of Fenpiverinium, Pitofenone and Diclofenac were prepared in linearity range and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using following equations. LOD = (3.3 ×Syx)/b, LOQ= (10.0×Syx)/where Syx is residual variance due to regression; b is slope.

Robustness: It helps in determining the reliability of the method. Fenpiverinium, Pitofenone and Diclofenac robustness is performed by deliberately changing the chromatographic conditions. The organic strength was varied by ±5%, column temperature was varied by ±5^{0}c and the flow rate was varied by ±0.1mL.

Stability: The sample and standard solutions are injected at 0 hr (comparison sample) and after 24 hr (stability sample) by keeping at ambient room temperature. Stability was determined by determining %RSD for sample and standard solutions.

***Degradation studies*:**
Oxidation:

To 1 ml of stock solution of Fenpiverinium, Pitofenone and Diclofenac, 1ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^{0}c. For HPLC study, the resultant solution was diluted to obtain 1µg/ml, 50µg/ml & 500µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

***Acid Degradation Studies*:**

To 1 ml of stock s solution Fenpiverinium, Pitofenone and Diclofenac, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^{0}c .The resultant solution was diluted to obtain 1µg/ml, 50µg/ml & 500µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1 ml of stock solution Fenpiverinium, Pitofenone and Diclofenac, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^{0}c. The resultant solution was diluted to obtain 1µg/ml, 50µg/ml & 500µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105 °C for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 1µg/ml, 50µg/ml & 500µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 10µg/ml, 500µg/ml & 5000µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 1µg/ml, 50µg/ml & 500µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60ºc. For HPLC study, the resultant solution was diluted to 1µg/ml, 50µg/ml & 500µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Statistical analysis:

Wherever applicable, results were expressed as the Mean±SD, %RSD and data were analyzed statistically by using t- test with aid of Microsoft excel-2007 software and data were considered not significantly different at 5% significance level of probability P ≤0.05**.**

III. RESULTS AND DISCUSSION

Fenpiverinium, Pitofenone and Diclofenac method development:

Initially reverse phase liquid chromatography separation was tried to develop using various ratios of Methanol and Water, Acetonitrile and water as mobile phases, in which drugs did not responded properly, and the resolution was also poor. The organic content of mobile phase was also investigated to optimize the separation of drugs. To improve the tailing factor, the pH of mobile phase becomes important factor. Discovery 250mm x 4.6 mm, 5µ with a isocratic mobile phase composed of Ortho phosphoric acid Acetonitrile (50:50A) at a flow rate of 1 mL/min. The column temperature was maintained at 30°C and the detection was carried out using a PDA detector at 241 nm, was selected as the stationary phase to improve resolution and the tailing of both peaks were reduced considerably and brought close to 1. To analyze drug detection were tried at wavelengths 241 nm. Fenpiverinium, Pitofenone and Diclofenac showed maximum absorption at 241 nm of wavelength and 241 nm was selected as the detection wavelength for PDA detector. The retention times were found to about 2.622 min, 3.172 min. and 3.838 min for Fenpiverinium, Pitofenone and Diclofenac. The chromatogram obtained was shown in the Fig. 1.

Fenpiverinium, Pitofenone and Diclofenac and met method Validation:

System suitability and Specificity: System suitability parameters such as number of theoretical plates, peak tailing, and retention time was determined. The total run time required for the method is only 6 minutes for eluting Fenpiverinium, Pitofenone and Diclofenac. The results obtained were shown in Table No.1. The chromatogram obtained for blank and spiked was shown in the Fig. 2.

Linearity: Fenpiverinium, Pitofenone and Diclofenac showed a linearity of response between 0.25-1.50 µg/mL for Fenpiverinium, 12.5 -7575µg/mL for Pitofenone and 125 - 750µg/mL for Diclofenac. These were represented by a linear regression equation as follows: y (Fenpiverinium, Pitofenone and Diclofenac) = 15666x + 129.7, 14848+886.5 & 3617+287.9 (r^2=0.999), (r2=0.999) and regression line was established by least squares method and correlation coefficient (r^2) for Fenpiverinium, Pitofenone and Diclofenac is found to

be greater than 0.98. Hence the curves established were linear.

Accuracy: To pre analyzed sample solution, a definite concentration of standard drug (50%, 100% & 150 % level) was added and recovery was studied. The % Mean recovery for Fenpiverinium, Pitofenone and Diclofenac are 100.30%, 99.47% and 100.02%. These results are within acceptable limit of 98-102. The % RSD for Fenpiverinium, Pitofenone and Diclofenac was 1.01, 0.5 and 0.4 and %RSD for Fenpiverinium, Pitofenone and Diclofenac is within limit of ≤2, hence the proposed method is specific and the results were summarized in Table No.2.

Precision: Repeatability: Six replicates injections in same concentration (1µg/ml, 50µg/ml & 500 µg/ml of Fenpiverinium, Pitofenone and Diclofenac) were analyzed in the same day for repeatability and the % RSD for Fenpiverinium, Pitofenone and Diclofenac found to be 0.9, 0.83 and 0.80. The % RSD for Fenpiverinium, Pitofenone and Diclofenac found to be within acceptable limit of ≤2 and hence method is reproducible and the results are shown in Table No. 3**.**

Intermediate Precision: Six replicates injections in same concentration were analyzed on two different days with different analyst and column for verifying the variation in the precision and the % RSD for Fenpiverinium, Pitofenone and Diclofenac is found to be 0.9, 0.5 and 0.80 and it is within acceptable limit of ≤2. Hence the method is reproducible on different days with different analyst and column. This indicates that the method is diplomatic and the results are as shown in Table No.4.

Robustness*:* The robustness was established by changing the flow rate, column temperature and composition of the mobile phase within allowable limits from actual chromatographic conditions. It was observed that there were no marked change in mean R_t and RSD is within limit of ≤2 .The tailing factor, resolution factor and no. of theoretical plates are found to be acceptable limits for Fenpiverinium, Pitofenone and Diclofenac. Hence the Method is reliable with variations in the analytical conditions and the results of Fenpiverinium, Pitofenone and Diclofenac was shown in Table No.5.

Stability of sample solution: The sample solution injected after 24 hr by keeping at ambient room temperature 30°C did not show any appreciable change. The % Deviation in the assay is not more than 2 and the results are shown in table-6.

LOD and LOQ: LOD and LOQ for Fenpiverinium, Pitofenone and Diclofenac were 0.003, 0.03&0.13 and 0.01, 0.08 & 0.41 µg/mL respectively. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive.

Tablet Analysis: The Content of Fenpiverinium, Pitofenone and Diclofenac in the tablets was found by the proposed method. RSD values for Fenpiverinium, Pitofenone and Diclofenac was within limit of ≤2 and the results were shown in Table No. 7.

Degradation studies: The degradation studies for Fenpiverinium, Pitofenone and Diclofenac was performed by various conditions like Acid, Alkali, Oxidation, Thermal, Photolytic and Neutral Degradation Studies and their limits like purity angle and purity threshold values like purity angle< purity threshold and the results shown in table no.8.

IV. CONCLUSION

As this method is precise, accurate simple and successful it is used for development and validation of Fenpiverinium, Pitofenone and Diclofenac pharmaceutical dosage form basing on ICH guidelines. In this method compound is degraded by oxidation, acid, alkali and by sunlight and analysed.It helps in improving the quality of the compound quantitatively and qualitatively. Hence it can be employed for routine quality control of tablet containing drug in QC laboratories and industries.

ACKNOWLEDGMENT

We, the authors, express our sincere gratitude to management, J.K.C. College, Guntur, and members of department of chemistry Acharya Nagarjuna University, Guntur, for the laboratory facilities provided to conduct this research work.

REFERENCES

[1] R. G Chatwal, Anand K.S. High performance liquid chromatography, 5th ed; Himalaya publishers: Mumbai, 2010; 2.570-2.629.

[2] B. K Sharma, High performance liquid chromatography, 24th ed; Goel publishers: Meerut, 2005; 295-300.

[3] W.M. Dong HPLC Instrumentation and trends. USA. 2006; 5-10, 78-110.

[4] A. Skoog, DM West, FJ Holler, 7th edition, Saunders College Publishing, Philadelphia, 1992, P.1-3.

[5] K. A Corners, A Wiley- inter science Publication, 1st edition 1967, P.475-478.

[6] A.V Kasture., Wadodkar S.G., Mahadik K.R., More H.N. Published by Nirali Prakashan, 13th edition, 2005

[7] A.H. Beckett and Stanlake ,Part 2, CBS Publishers and Distributors; 4th edition.2002; P.157-174.

[8] R.L Snyder, kirklannd J.J, Glaijch, 2nded; new York, 1997; 30-100.

[9] A. Satinder, Dong M.W, Pharmaceutical analysis by HPLC, 15th ed; Newyork, 2005; 16-70.

[10] M.E Swartz, Ira krull, 1sted; Marcel Dekker, New York, 2009; 17-80.

[11] Rahul Singh1 et al ., (2013) reported as Method Indian J.Pharm.Biol.Res. 2013 ; 1(4):95-101

[12] Mehul m patel et al ,.(2012) Asian journal of research in chemistry 05/2012; 5(5):698.

[13] S. K. Dubey et al ,. (2013) Indian J Pharm Sci. 2013 Jan-Feb; 75(1): 89–93.

[14] Muhammad Ashfaq,*b et al ,.(2012), 4, 2121-2126 Chromatography Research International Volume 2013 (2013), Article ID 424865, 6 pages

[15] A.P. Dewania et al ,. (2014) Arabian Journal of Chemistry Volume 7, Issue 5, November 2014, Pages 811–816

[16] Kumar A1 et al ,. (2012) Acta Pol Pharm. 2012 Nov-Dec;69(6):1017-22.

[17] Atul Nikam* et al ,.(2013), IAJPR. 2013; 3(6): 4330-4338

[18] Rouhollah_Heydari et al ,.(2008) Pharmaceutical Formulations in Analytical Letters (Impact Factor: 0.98). 05/2008; 41(6):965-976. DOI: 10.1080/00032710801978137

[19] Boyka G. Tsvetkova* et al ,. (2012) Der Pharmacia Sinica, 2012, 3 (4):400-403

[20] Bhaskar daravath et al ,. (2014) Asian journal of pharmaceutical and clinical research Vol 7, Issue 3, 2014 ISSN - 0974-2441

Fig.1. Chromatograms of standard

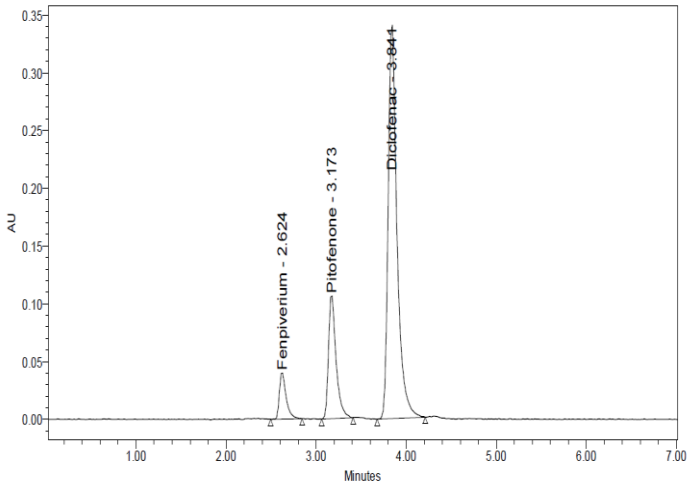

Fig.2. Chromatograms of sample

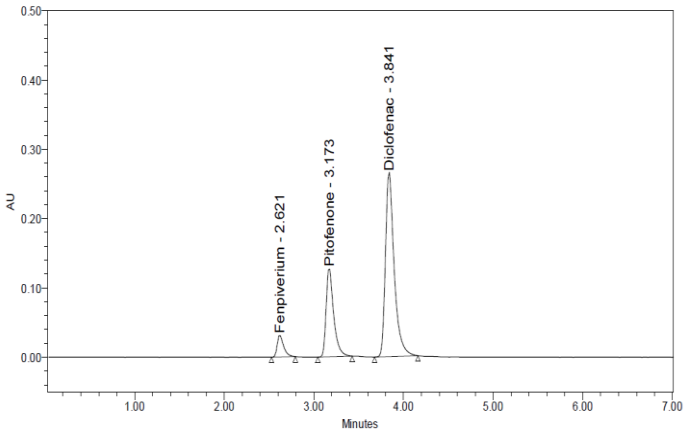

Fig.3. Chromatograms of Blank

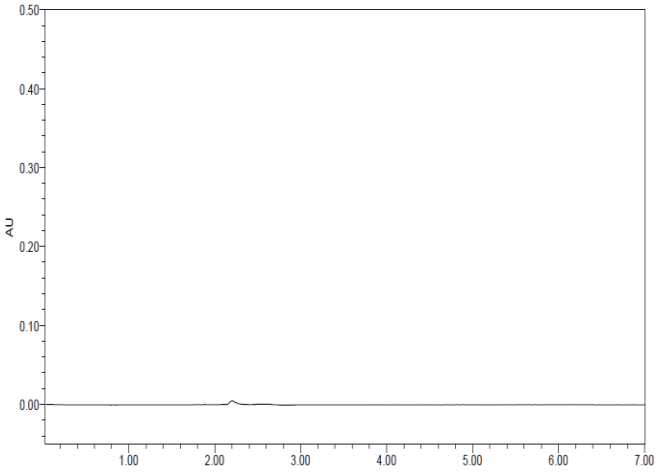

Fig.4. Placebo chromatogram

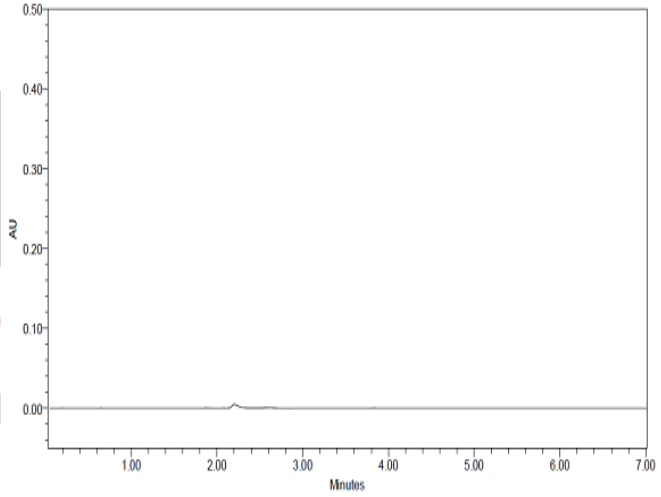

Fig.5. Degradation overlay chromatogram

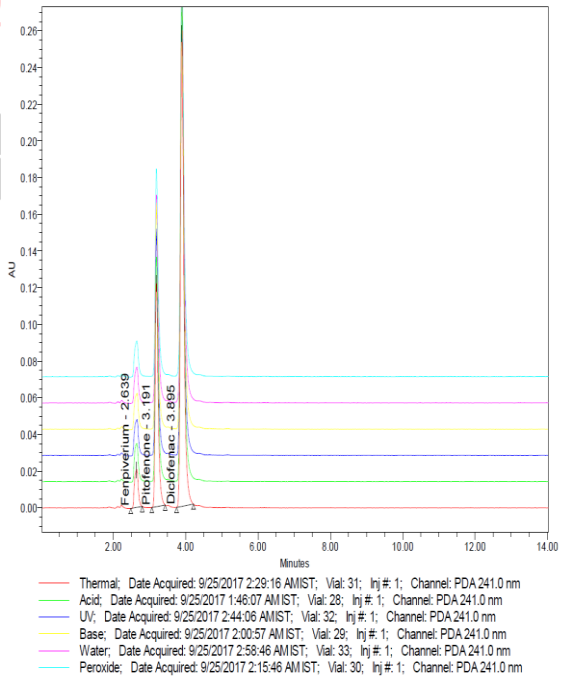

Table No.1: System suitability of Fenpiverinium, Pitofenone and Diclofenac

S no	Pitofenone			Fenpiverinum			Diclofenac		
Inj	RT(min)	TP	Tailing	RT(min)	TP	Tailing	RT(min)	TP	Tailing
1	3.171	7467	1.49	2.621	6495	1.39	3.835	8307	1.47
2	3.171	7601	1.49	2.621	6647	1.38	3.835	8346	1.47
3	3.172	7505	1.47	2.622	6425	1.44	3.838	8439	1.46
4	3.172	7344	1.50	2.622	6462	1.45	3.838	8022	1.47
5	3.172	7456	1.48	2.622	6646	1.47	3.841	8109	1.46
6	3.172	7927	1.48	2.622	6249	1.44	3.841	8199	1.46

Table No.2: Results of accuracy of Fenpiverinium, Pitofenone and Diclofenac

Sample name	% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
Fenpiverinium	50%	0.5	0.501	100.18	100.30%
		0.5	0.498	99.70	
		0.5	0.496	99.24	
	100%	1.0	1.018	101.82	
		1.0	1.003	100.32	
		1.0	1.007	100.68	
	150%	1.5	1.488	99.18	
		1.5	1.520	101.35	
		1.5	1.503	100.21	

Sample name	% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
Pitofenone	50%	25	25.00	99.98	99.47%
		25	24.80	99.20	
		25	25.06	100.25	
	100%	50	49.51	99.01	
		50	49.84	99.69	
		50	49.87	99.75	
	150%	75	74.32	99.09	
		75	74.48	99.30	
		75	74.25	99.00	

Sample name	% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
Diclofenac	50%	250	249.91	99.97	100.02%
		250	254.04	101.62	
		250	248.94	99.58	
	100%	500	496.44	99.29	
		500	499.30	99.86	
		500	500.41	100.08	
	150%	750	749.76	99.97	
		750	751.41	100.19	
		750	747.10	99.61	

Table No.3: Results of Precision for Fenpiverinium, Pitofenone and Diclofenac

S. No	Area of Pitofenone	Area of Fenpiverinium	Area of Diclofenac
1.	737344	15588	1773819
2.	736146	15550	1808119
3.	738093	15682	1778553
4.	736432	15685	1778952
5.	748894	15811	1787035
6.	747506	15880	1803014
Mean	740736	15699	1788249
S.D	5839.0	126.7	14163.1
%RSD	0.8	0.8	0.8

Table No. 4: Results of Intermediate precision table of Pitofenone, Fenpiverinium and Diclofenac

S. No	Area of Pitofenone	Area of Fenpiverinium	Area of Diclofenac
1.	773709	15235	1753213
2.	773135	15337	1774223
3.	764236	15568	1754975
4.	770763	15290	1741092
5.	774736	15243	1769801
6.	774183	15499	1742105
Mean	771794	15362	1755902
S.D	3950.1	139.5	13759.5
%RSD	0.5	0.9	0.8

Table 5: Results of Robustness for Fenpiverinium, Pitofenone and Diclofenac

Table 5: Results of Robustness for Fenpiverinium, Pitofenone and Diclofenac

S.no	Condition	%RSD of Pitofenone	%RSD of Fenpiverinium	%RSD of Diclofenac
1	Flow rate (-) 0.9ml/min	0.5	0.6	0.3
2	Flow rate (+)	0.3	0.5	0.3
3	Mobile phase (-)	0.8	1.24	0.8
4	Mobile phase (+)	0.8	0.88	1.0
5	Temperature (-) 25°C	1.6	0.41	0.1
6	Temperature (+) 35°C	0.4	0.68	0.7

Table 6: Assay Data of Fenpiverinium

S.no	Standard Area	Sample area	% Assay
1	15474	15588	99.46
2	15446	15550	99.22
3	15827	15682	100.06
4	15759	15685	100.08
5	15571	15811	100.88
6	15679	15880	101.32
Avg	15626	15699	100.17
Stdev	154.5	126.7	0.809
%RSD	1.0	0.8	0.81

Assay Data of Pitofenone

S.no	Standard Area	Sample area	% Assay
1	745132	737344	99.56
2	742445	736146	99.40
3	735125	738093	99.66
4	737567	736432	99.44
5	738644	748894	101.12
6	735752	747506	100.93
Avg	739111	740736	100.02
Stdev	3927.9	5839.0	0.79
%RSD	0.5	0.8	0.79

Assay Data of Diclofenac

S.no	Standard Area	Sample area	% Assay
1	1798695	1773819	99.16
2	1795249	1808119	101.08
3	1781824	1778553	99.42
4	1783788	1778952	99.44
5	1781404	1787035	99.90
6	1781613	1803014	100.79
Avg	1787096	1788249	99.96
Stdev	7774.4	14163.1	0.792
%RSD	0.4	0.8	0.79

Table 7: Results of Sensitivity table of Pitofenone, Fenpiverinium and Diclofenac

Molecule	LOD(µg/ml)	LOQ(µg/ml)
Pitofenone	0.03 µg/ml	0.08 µg/ml
Fenpiverinium	0.003 µg/ml	0.01µg/ml
Diclofenac	0.13 µg/ml	0.41 µg/ml

Table 8: Results of HPLC Degradation of Fenpiverinium, Pitofenone and Diclofenac Degradation Data of Fenpiverinium

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	6.28	0.153	0.342
2	Alkali	7.27	0.147	0.336
3	Oxidation	5.36	0.153	0.342
4	Thermal	3.73	0.145	0.338
5	UV	3.07	0.147	0.336
6	Water	0.89	0.145	0.338

Degradation Data of Pitofenone

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	5.02	0.626	0.827
2	Alkali	6.30	0.612	0.792
3	Oxidation	6.56	0.626	0.827
4	Thermal	5.02	0.652	0.815
5	UV	3.60	0.617	0.806
6	Water	0.75	0.652	0.815

Degradation Data of Diclofenac

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	5.98	0.099	0.304
2	Alkali	3.78	0.097	0.300
3	Oxidation	4.42	0.099	0.303
4	Thermal	3.74	0.090	0.302
5	UV	1.55	0.097	0.300
6	Water	0.48	0.090	0.302